EXPERIMENTS WITH METHODS FOR THE RAPID DETECTION OF GELATIN LIQUEFACTION IN THE DETERMINATION OF *B. COLI*.

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In determining the presence or absence of bacteria of the colon type by the methods approved by the Committee on Standard Methods of Water Analysis, the procedure consists essentially of three steps: first, a detection of gas-producing organisms by means of a fermentation test; second, the separation of acid-fermenting types from other types by plating on litmus-lactose agar and the transfer of typical colonies to agar streaks; third, the identification of the cultures so isolated by their cultural and biochemical reactions when grown in dextrose broth, milk, nitrated peptone solution, Dunham's solution, and gelatin. Since cultures of the colon type grow rapidly and luxuriantly at 40° C., it is possible to complete the first two steps and the identification tests for fermentation, milk coagulation, and nitrate reduction within four days, and the indol test within seven days from the time that the examination of the sample was begun. Since the ordinary culture gelatin does not remain solid at temperatures as high as 40° C., and as the liquefaction of gelatin proceeds very slowly in the case of many common types of bacteria, which can best be excluded from the colon type by this test, it is customary to incubate the gelatin cultures at 20° C. for a period of 14 days. The delay of 10 days required to obtain the liquefaction tests after the other tests are complete is objectionable in many cases where it is desirable that analyses should be reported at the earliest possible date, and many methods have been proposed by which this delay might be obviated. A number of these methods, all based upon the assumption that because the types of bacteria commonly encountered in testing for *B. coli* grow more rapidly and more luxuriantly at body temperature than at room temperature the production of proteolytic enzymes will be correspondingly rapid at the higher temperature, have been investigated at the Lawrence Experiment Station during the past eight

years. Since data which tend to disprove this fundamental assumption must apply with equal force to all the methods tested, statistics regarding only one of the methods will be presented, together with a general statement of the results obtained in tests of the other methods. It is not the purpose of the present paper to enter into a general consideration of the properties of bacterial enzymes, or the conditions under which those properties are manifest, except in so far as they may apply to the specific problem of obtaining a rapid diagnosis of gelatin liquefaction in routine *B. coli* tests.

Substitutes for gelatin.—The three media commonly used for liquefaction tests in bacteriology are gelatin, blood serum, and casein. At the suggestion of the late Dr. Wyatt Johnston, the writer studied the possibility of substituting liquefaction tests on Löffler's blood serum at body temperature for gelatin tests at room temperatures some years ago. In that investigation it was found that a considerable percentage of cultures liquefying gelatin in 14 days at 20° C. failed to liquefy serum at 40° C. in four days or less, although a luxuriant growth usually occurred in 18 to 24 hours. In an investigation of the species of bacteria commonly found associated with *B. coli* at Lawrence, out of 46 different species isolated and studied, six only were found which liquefied both gelatin and serum, while 12 liquefied gelatin but did not liquefy serum, and five liquefied serum but did not liquefy gelatin. In other words, we would have obtained a correct report with only 13 per cent of the species, from liquefaction tests on serum, when the tests were made at 20° C., and it is quite probable that a shorter incubation at 40° C. would have shown even a smaller percentage accuracy.

The great difficulty in preparing a casein medium upon which liquefaction tests can be accurately made is a serious objection to its use. No direct investigation of casein as a substitute for gelatin has been made at Lawrence. In the study of species of bacteria mentioned above, however, only five species liquefied both casein and gelatin, and 13 species which liquefied gelatin failed to liquefy casein.

The investigations of Malfitano,[1] published in 1903, definitely established the fact that the liquefaction of albumen and of gelatin

[1] *Compt. rend. de la Soc. de Biol.*, 1903, 55, p. 843.

was due to entirely separate and distinct enzymes, and show the futility of attempting to substitute serum or casein for gelatin.

Modified gelatin.—A number of processes for preparing a gelatin medium which should remain solid at 40° C. and at the same time embody the properties of the ordinary culture gelatin have been tried.

Mixtures of gelatin and agar were first proposed by Hiss[1] for separating the typhoid, colon, and paracolon types. Attempts to use media of this type for liquefaction tests at 40° C. proved unsuccessful for the reason that if the proportion of agar present was great enough to make the media even reasonably stiff, liquefaction occurred very slowly or not at all, while a fluid or semifluid mixture had no advantages over gelatin alone.

Mixtures of gelatin and serum were suggested by the gelatin-agar mixtures. A number of such mixtures were tried with little success, cultures which liquefied gelatin at 20° C. frequently failing to show any indication of liquefaction when grown on these media for four days at 40° C. Any liquefaction results obtained on such media would, of course, be subject to the same inaccuracy as in the use of serum alone.

In 1901 a German investigator[2] suggested that the melting-point of gelatin might be raised to above 40° C. by the addition of small quantities of formalin without impairing its value as a medium for the cultivation of bacteria. Many attempts have been made at Lawrence to prepare a satisfactory medium in this manner. The problem is to add just the proper amount of formalin. If too much be added the gelatin becomes very hard and liquefaction proceeds very slowly, any considerable excess of formalin will effectually prevent the development of bacterial growth, while if too little be added the melting-point will not be raised sufficiently to prevent the gelatin becoming fluid at 40° C. The process might possibly be worked on a commercial scale to produce a bacteriological gelatin, but its application in the laboratory appears to be impractical.

Attempts to prepare gelatin media with a melting-point between 40° and 50° C. by the addition of alcohol or tannic acid were also attended with little success, the difficulties encountered being similar to those in the formalin process.

[1] *Jour. Med. Res.*, 1902, 8, p. 148.

[2] *Centralbl. f. Bakt.*, 1901, 30, Abt. 1, p. 368.

Preliminary cultivation methods.—The small success obtained in the experiments previously described led to a radical change in procedure. Cultures of liquefying bacteria were seeded into broth and incubated 24 to 48 hours at 40° C., 1 c.c. of the luxuriant culture so obtained was mixed with 5 c.c. of fluid gelatin, and, after solidifying the gelatin, the culture was incubated at 20° C. By such procedure it was hoped that the introduction of a large number of bacteria, together with whatever enzymes might have been formed in the broth, would result in liquefaction becoming manifest in a shorter period than would be the case when stab cultures were used.

The conclusions of Brunton and Macfadyen,[1] that enzymes produced in meat broth liquefied gelatin more rapidly than did those produced in gelatin itself, would lend credence to the advisability of such a procedure.

Cultures started in this way quite uniformly liquefied one to two days earlier than did stab cultures made at the time that the broth cultures were mixed with the gelatin, showing that greater bacterial activity had resulted from the introduction of large numbers of bacteria into the gelatin, but the time required to prepare the "starter," together with the time required to obtain the liquefaction test in the enriched gelatin culture, was about the same as that required for a similar test with stab cultures direct.

A modification of the preceding method consisted in incubating a broth culture of the test organism for four days at 40° C. and then testing for the presence of liquefying enzymes by pouring the culture on the surface of gelatin after marking the level of the gelatin surface, determining whether liquefaction ensued by any change in the depth of the gelatin. This method proved to be less efficient as a means of rapid liquefaction diagnosis than was the preceding one.

Fermi[2] states that the production of gelatinase is more abundant in gelatin media than in any other media and his statement was confirmed by Abbott and Gildersleeve.[3] In the recent experiments of Jordan[4] it was found that the rate of enzyme production during the first 10 days with seven different rapidly liquefying species, was more

[1] *Proc. Roy. Soc.*, 1889, 46, p. 543.

[2] *Centralbl. f. Bakt.*, 1890, 7, p. 469.

[3] *Jour. Med. Res.*, 1903, 10, p. 42.

[4] *Biological Studies by Pupils of Wm. T. Sedgwick*, University of Chicago Press, 1906, p. 127.

rapid in broth than in gelatin at 37.5° C. in four out of seven experiments, while it was more rapid in broth at 20° C. in only two out of seven experiments.

Gelatin at 20° and 40° C.—The results obtained in the preceding experiments having shown that it was futile to attempt to obtain early liquefaction results by cultivation in broth, studies were commenced in which gelatin cultures were incubated at 40° C., the gelatin of course being fluid at that temperature. After some preliminary experiments, a routine procedure was adopted as follows: Cultures which had shown the power to liquefy gelatin at 20° C. during the regular *B. coli* tests, were transferred to agar streaks, grown over night at 40° C., and were then reinoculated into gelatin tubes and placed in the 40° C. incubator for four days. The fluid gelatin cultures so obtained were removed at the end of 24 hours, two days, three days, and four days, and placed on ice for one hour, after which treatment those tubes in which the gelatin had solidified were returned to the incubator and the cultures in tubes in which the gelatin failed to solidify in one hour on ice were recorded as having produced liquefaction.

The investigation extended over a period of 10 months ending November 1, 1903. During this time about 2,800 cultures obtained in routine *B. coli* determinations from samples from a variety of sources were submitted to the gelatin liquefaction test by the standard procedure, and 368, or 13 per cent of these cultures, were found to show signs of liquefaction in 14 days or less at 20° C.; 288 cultures, selected at random from among these liquefying cultures, were tested for liquefaction at 40° C. by the method described above. In the routine liquefaction tests at Lawrence the approximate time required for liquefaction to ensue is shown by readings made on the fourth, seventh, tenth, and fourteenth days, and the type of liquefaction has always been recorded, three primary types of liquefaction being recognized: the stratiform type, the funnel, including the saccate and infundibuliform types, and the cup, including the craterform and the napiform types. The detailed results of the tests at 40° C. are shown in Table 1, the cultures being grouped according to their variations at 20° C.

Of the 288 cultures tested by both methods liquefaction developed

TABLE 1.

RESULTS OF TESTS FOR LIQUEFACTION AT 20° C. AND AT 40° C.

GELATIN AT 20° C.			GELATIN AT 40° C.					
Type of Liquefaction	Date Liquefaction Was First Noted	Number of Cultures	Number of Cultures First Showing Liquefaction on				Total Number of Cultures	
			First Day	Second Day	Third Day	Fourth Day	Liq.	Non-Liq.
Cup	4th day	9	3	2	1	0	6	3
	7th	6	1	0	0	0	1	5
	10th	17	3	0	0	0	3	14
	14th	121	18	0	0	4	22	99
Funnel	4th	48	33	1	1	1	36	12
	7th	10	0	0	0	0	0	10
	10th	17	2	1	0	0	3	14
	14th	31	6	1	0	2	9	22
Stratiform	4th	21	12	3	1	0	16	5
	7th	3	1	0	0	0	1	2
	10th	2	1	0	1	0	2	0
	14th	3	0	0	0	0	0	3

at 20° C. in 27 per cent in four days or less, in 7 per cent between the fourth and seventh days, in 12 per cent between the seventh and tenth days, and in 54 per cent between the 10th and 14th days, 53 per cent of the liquefactions being of the cup type, 37 per cent of the funnel type, and 10 per cent of the stratiform type.

The rate of liquefaction at 20° C. by cultures producing the cup type of liquefaction was very different from that of cultures producing the funnel and stratiform types, 73 per cent of the stratiform liquefactions and 45 per cent of the funnel liquefactions being manifest within four days, while only 6 per cent of the cup liquefactions were noted within the same period. Furthermore, 79 per cent of the cup liquefactions, 29 per cent of the funnel liquefactions, and 10 per cent of the stratiform liquefactions were not manifest until after the 10th day. We thus observe that a shortening of the time of incubation of gelatin cultures to 10 days or less, as has been the practice in some laboratories, would result in the exclusion of four-fifths of the cup liquefiers, and from one-tenth to one-fourth of the other forms from among the liquefying types, with a consequent increase in the reported presence of *B. coli* in samples from which it was undoubtedly absent. The records of the examination of over 23,000 cultures obtained in routine *B. coli* determinations during the past five years, amply substantiate the statement that a shortening of the gelatin incubation period would introduce a serious error into the analytical results.

An examination of the results obtained at 40° C. reveals the fact that 28 per cent of all the cultures tested produced liquefaction within 24 hours, and 34 per cent within four days, while 66 per cent of the cultures failed to produce any signs of liquefaction whatever during the period of four days that they were under observation. A certain similarity between the behavior of cultures of the cup, funnel, and stratiform types at 20° and at 40° C. is also apparent; 65 per cent of the cultures of the stratiform type and 46 per cent of those of the funnel type showed liquefaction at 40° C. within four days, while only 21 per cent of the slowly liquefying cup type were able to produce liquefaction at the higher temperature within the same period. About four-fifths of all cultures which were able to produce peptonization at all during four days at 40° C. did so within the first 24 hours. The rate of liquefaction at 20° C. and at 40° C. by cultures of these different types is shown in the following table.

TABLE 2.

RELATIVE LIQUEFACTION AT 20° C. AND 40° C. BY CULTURES OF THE CUP, FUNNEL, AND STRATIFORM TYPES.

		All Cultures	Cup Type	Funnel Type	Stratiform Type
Number of cultures		288	153	106	29
Per cent. of cultures first showing liquefaction at 20° C. on the	4th day	27	6	45	73
	7th "	7	4	10	10
	10th "	12	11	16	7
	14th "	54	79	29	10
Per cent of cultures first showing liquefaction at 40° C. on the	1st "	28	16	39	48
	2d "	3	1	3	10
	3d "	1	1	1	7
	4th "	2	3	3	0
Per cent of cultures liquefying at 40° C.		34	21	46	65
Per cent of cultures not liquefying at 40° C.		66	79	54	35

There is apparently very little correlation between the rate of liquefaction at 20° C. and liquefaction at 40° C. To be sure, a much greater proportion of the cultures which had produced liquefaction at 20° C. within four days reacted within a like period at 40° C. than was the case with cultures which produced gelatinase more slowly at the lower temperature. For example, 74 per cent of the four-day 20° C. cultures liquefied within four days at 40° C., while only 11 per cent, 22 per cent, and 20 per cent, respectively, of the 7-, 10-, and 14-day cultures reacted within the same period. That is to say, about one-fifth of the cultures which were unable to produce lique-

faction within one week at 20° had their peptonizing function so accelerated by incubation at 40° that they were able to react within four days. On the other hand, about one-fourth of the cultures which liquefied within four days at 20° C. were unable to produce a like reaction in the same period at 40° C., although taking all the cultures into consideration there was an increase of about 6 per cent in liquefactions recorded within four days at 40° C. over those recorded in the same period at 20° C. The failure of so many cultures to produce peptonizing enzymes at 40° C. in the same time that they were able to do so at 20° C. is especially significant, as it indicates that the optimum temperature for enzyme production is quite different with different cultures, and further suggests that if this be the case, it is quite possible that by cultivation at the proper temperature, the liquefying function might be induced in many cultures which have hitherto been classed among the non-liquefying types. Jordan[1] has recently demonstrated that the optimum temperature for the reaction between the liquefying enzyme and the gelatin and the optimum temperature for the bacterial production of that enzyme are often widely different. The relative liquefaction at 40° C. by cultures which produced peptonization in 4, 7, 10, and 14 days, respectively, is shown in the following table.

TABLE 3.

LIQUEFACTION AT 40° C. BY CULTURES LIQUEFYING IN 4, 7, 10, AND 14 DAYS RESPECTIVELY AT 20° C.

		Fourth Day	Seventh Day	Tenth Day	Fourteenth Day
Number of cultures		78	19	36	155
Per cent of cultures first showing liquefaction at 40° C. on the	1st day	61	11	18	15
	2d "	8	0	2	1
	3d "	4	0	2	0
	4th "	1	0	0	4
Per cent of cultures liquefying at 40° C.		74	11	22	20
Per cent of cultures not liquefying at 40° C.		26	89	78	80

CONCLUSIONS.

The results of the foregoing experiments have been almost completely a record of failures, due undoubtedly to the fact that the fundamental assumption, that since the growth of bacteria of the types in question was more rapid at 40° C. than at 20° C., the produc-

[1] *Loc. cit.*

tion of liquefying enzymes must also be accelerated at the higher temperature, was untenable. We have learned by past experience that the other biochemical functions by whose aid we are able to separate from the colon group many types of foreign bacteria, the production of gas and acid in sugar solutions, the reduction of nitrates, the coagulation of milk, and the production of indol are more active at the higher temperature. We learn from these experiments, however, that the liquefying function does not as a rule follow the same law as the other biochemical functions.

Concerning the attempts to prepare a gelatin medium which should be solid at a temperature of 40° C. upon which liquefaction might be noted in the same manner as that employed with ordinary culture gelatin at 20° C., it need only be said that, while such media can be made, its preparation is difficult, and by the use of more recent methods of testing for liquefaction, its use becomes unnecessary in dealing with the specific problem—the shortening of the time involved in making tests for *B. coli.*

The use of broth cultures to accelerate the production of liquefying enzymes also appears to be of doubtful value. From the comparative experiments with gelatin cultures at 20° C. and at 40° C. we see that more than half of the liquefying cultures commonly found during routine *B. coli* tests at Lawrence were of one type, with about four-fifths of which liquefaction was not manifest until after the 10th day, and with this type of cultures liquefaction was not produced within four days, when they were incubated at 40° C. Furthermore, we note that a very considerable proportion of cultures of the funnel and stratiform types, which usually produce liquefaction within four to seven days at 20° C., failed to show liquefaction within four days at 40° C. That is to say, while the activity of the liquefying function was greatly increased at the higher temperature in some instances, it was in other cases materially retarded.

The temperature at which the liquefying enzymes act most energetically upon gelatin has been determined by a number of investigators. The determination of the temperature at which the greatest bacterial activity in enzyme production shall be combined with the maximum liquefying activity of that enzyme appears, however, to have been overlooked, and it appears to the writer that data of this

character are necessary before we may formulate a method for determining liquefaction which shall be at the same time rapid and accurate. The preparation of media more favorable for rapid enzyme production than are the usual culture media, and the establishment of more refined chemical tests for the detection of minute quantities of the liquefying enzymes would also appear to be fertile fields for investigation.